Sampling strategies for wastewater surveillance: Evaluating the variability of SARS-COV-2 RNA concentration in composite and grab samples

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ABSTRACT

The shedding of SARS-CoV-2 RNA titers by infected individuals, even asymptomatic and oligosymptomatic ones, allows the use of wastewater monitoring to track the COVID-19 spread in a community. This approach is interesting especially for emerging countries with limited clinical testing capabilities. However, there are still important methodological aspects that need validation so that wastewater monitoring data become more representative and useful for public health. This study evaluated the between-day and within-day variability of SARS-CoV-2 RNA concentrations in 24-hour composite and grab samples from three different sampling points, including two wastewater treatment plants (WTTP) and a sewer manhole. In the between-day evaluation (17 weeks of monitoring), a good agreement between the SARS-CoV-2 RNA concentration of each sampling method was observed. There were no significant differences between the mean concentrations of the grab and composite samples (p-value > 0.05), considering N1 and N2 gene assays. The strong relationship between composite and grab samples was proven by correlation coefficients: Pearson's r of 0.83 and Spearman's rho of 0.78 (p-value < 0.05). In within-day evaluation, 24-hour cycles were analyzed and low variability in hourly viral concentrations was observed for three sampling points. The coefficient of variation (CV) values ranged from 3.0% to 11.5%. Overall, 24-hour profiles showed that viral RNA concentrations had less variability and greater agreement with the mean values between 8 a.m. and 10 a.m, the recommended time for grab sampling. Therefore, this study provides important information on wastewater sampling techniques for COVID-19 surveillance. Wastewater monitoring information will only be useful to public health and decision-makers if we ensure data quality through best practices.

1. Introduction

The novel coronavirus, SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2), first detected in Wuhan, China, in December 2019, has been identified as the etiological agent of COVID-19 (Corona Virus Disease 2019) pandemic. According to WHO [1], as of November 15th, 2021, there were 256,637,065 confirmed cases of COVID-19 worldwide, including 5,148,221 deaths. Brazil alone registered a total of 22,012,150 cases with 612,587 deaths.

COVID-19 patients may exhibit different symptoms such as dry cough, fever, shortness of breath, headache, diarrhea, among others.

These symptoms usually appear 2–14 days after contact with the virus. However, around 35.1% (95% CI: 30.7–39.9%) of infected individuals may be asymptomatic and never develop clinical symptoms [2]. Furthermore, clinical tests, which are scarce in low-income countries, are not completely reliable [3]. Therefore, there are significant uncertainties in the epidemiological data available, which must be considered by decision-makers.

Studies suggested that more than 41% of infected patients shed SARS-CoV-2 RNA in their stool [4,5], although the RNA shedding rate appears to be independent of the severity of the infection [6]. Wölfel et al. [7], for example, determined a shedding rate greater than 10^7 RNA

copies.g feces[-1] one week after symptom onset. SARS-CoV-2 RNA was also detected in stool samples from infected individuals whose clinical tests were negative [8]. Thus, several studies have also reported the presence of SARS-CoV-2 RNA titers in municipal wastewater samples [9–12] and the potential use of sewage monitoring as a complementary tool for public health surveillance for COVID-19 [3,13–18].

Wastewater surveillance is a tool originally designed to monitor the use of illicit drugs in a community and it has been successfully used for predicting the outbreak of Aichi virus in The Netherlands [19] and poliovirus in Israel [20]. The wastewater monitoring data could complement epidemiological/clinical data to provide a robust tool for monitoring an infectious disease [9,21–24]. While clinical data provides pooled individual information that is often difficult to interpret, wastewater provides an aggregated sample of an entire area. In addition to reducing monitoring costs, this approach allows the tracking of asymptomatic and oligosymptomatic individuals, who are generally not detected during clinical surveillance [25–28].

However, as attested by Shah et al. [29], the data published so far are insufficient to consolidate SARS-CoV-2 monitoring via wastewater surveillance. There are important methodological aspects to be validated and optimized such as sampling strategies and experimental methods (concentration and quantification of SARS-CoV-2 RNA) [21,30,31]. Inaccurate results of wastewater monitoring can lead to harmful decisions and inefficient interventions by authorities [32].

There are two major sampling methods: time- or flow-proportional composite sampling and grab sampling. A composite sample is a single sample volume constructed of multiple individual samples (aliquots) taken over a specific period. In time-proportional composite sampling, fixed volume aliquots are taken at uniform time intervals during the period of interest. A flow-proportional composite sampling can be performed using two different methods: i) by collecting a constant aliquot volume at varying time intervals proportional to the instantaneous wastewater flow rate; ii) by collecting aliquots of variable volume and proportional to the instantaneous wastewater flow rate, maintaining a constant time interval between the aliquots [33]. Composite sampling, usually performed over 24 h using manual or autosamplers, is highly recommended in wastewater monitoring. This sampling strategy is less susceptible to the inherent seasonal and diurnal variability of wastewater characteristics, including the viral content [34–36]. Several studies have reported the use of composite sampling in SARS-CoV-2 surveillance [10,11,13,26]. However, sampling periods longer than 6 h have been shown to be unfeasible, especially when it is necessary to collect samples from multiple locations and areas of difficult access (sewer manholes, rivers, among others) [37]. Furthermore, there is often a need for permanent care of sampling equipment, especially automatic samplers, to prevent theft. Thus, the use of grab sampling can be encouraged in some cases, even though it only represents the conditions of the exact moment of collection.

To date, the study of sampling strategies for SARS-CoV-2 surveillance using wastewater is limited [38–40]. Curtis et al. [39] found good agreement between the grab samples collected every 2 h in 72 h and three corresponding 24-hour flow-weighted composite samples. However, the authors suggested avoiding sample collection during periods of low flow and, consequently, of higher concentrations of potential inhibitory substances for PCR reactions. On the other hand, Gerrity et al. [40] verified a 10-fold increase in SARS-CoV-2 RNA concentrations in 24-hour flow-weighted composite sampling in comparison to corresponding grab sampling. Therefore, additional data are necessary for the standardization and validation of the sampling methods [30].

This study evaluated the variability of SARS-CoV-2 RNA concentrations in composite and grab samples of untreated wastewater from the ABC Region, São Paulo, Brazil. The differences between the two sampling methods were analyzed in two steps: (i) between-day evaluation (17 weeks of monitoring); (ii) within-day evaluation (two distinct 24-hour periods). In the second step, three different representative sampling points were assessed: (i) a large-scale wastewater treatment plant

(WWTP); (ii) a small-scale WWTP; (iii) a point in the sewer system (sewer manhole). This study provides important methodological information and insights for future wastewater surveillance research. The negligence of the sampling technique can greatly increase the uncertainty of monitoring data and, consequently, of COVID-19 prevalence estimates. Assuring data quality is essential to make wastewater surveillance information useful to decision-makers.

2. Material and methods

2.1. Sampling sites

The experiments were performed at three points of the ABC Region, São Paulo, Brazil: i) a large-scale WWTP; ii) a small-scale WWTP; iii) a point in the sewer system (sewer manhole). Untreated wastewater samples were collected and analyzed for SARS-CoV-2 RNA occurrence. The main information of sampling points is shown in Table 1.

2.2. Study design

This study evaluated the variability of SARS-CoV-2 RNA concentrations in composite and grab samples of untreated wastewater. The differences between the two sampling methods were analyzed in two steps: (i) between-day evaluation (17 weeks of monitoring); (ii) within-day evaluation (two 24-hour periods).

In the between-day evaluation step, only samples from point 1, a large-scale WTTP, were analyzed over 17 weeks (113 days), between April 14th, 2021 and August 4th, 2021. The 24-hour composite sampling (proportional to the hourly flow rate) was performed using a refrigerated Hach automatic sampler (model AWRS AS950), with a storage temperature of 4 °C. Grab sampling was always carried out between 8 a. m. and 10 a.m. on the same day as the composite sampling. For this time interval, there was a lower variability of SARS-CoV-2 RNA titers concentrations and a greater agreement with the average values, as will be discussed in Section 3.2 (within-day evaluation: 24-hour cycles).

In the within-day evaluation, grab samples were collected every hour over two 24-hour periods: day 1, a more severe moment of the pandemic, with a 14-day moving average around 600 new cases per day; and day 2, a milder moment, with a 14-day moving average around 300 new cases per day. In this step, the three different sampling sites (Table 1) were evaluated. For safety reasons, no samples were collected between 11 p.m. and 5 a.m. at point 3 (sewer manhole).

2.3. Viral detection

Viral particles were concentrated by the precipitation method, as described by Wu et al. [10]. Briefly, 40 mL of samples were centrifuged (8000xg/120 min/4 °C) and the pellet was resuspended in 0.4 mL of 1x PBS (pH 7.2). One milliliter of acid phenol was added and centrifuged (12.000xg/10 min/4ºC) for sample cleaning [13]. The aqueous phase was transferred to a microtube containing 0.3 mL of lysis buffer. RNA

Table 1
Sampling points characteristics.

Sampling point	Site category	Contributing population	Wastewater flow rate (L.s^{-1})
Point 1: WWTP ABC	Large-scale WWTP	1,400,000	2838.9[a]
Point 2: WTTP Parque Andreense	Small-scale WWTP	2320	2.0[a]
Point 3: Vila Vilma	Sewer system (sewer manhole)	2636	4.5[b]

[a] Measured (average value).
[b] Estimated (from population data and a per capita wastewater generation of 160 L.person[-1].d[-1]).

extraction was performed using the PureLink™ Viral RNA/DNA Mini Kit (Thermo Fisher Scientific), according to the manufacturer protocol. The final elution volume for the extraction was 80 μL.

To quantify SARS-CoV-2 RNA, reaction mixtures were prepared using the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Thermo Fischer Scientific, Waltham, MA, USA) for the targeted nucleocapsid (N1 and N2) genomic regions [41]. The enveloped bovine respiratory syncytial virus (BRSV – Inforce™ 3, Zoetis, US) was used for evaluation of concentration methods recovery capacity. The recovery rates were between 20% and 65% [13]. Primers and probes, with sequences and concentrations that are listed in Table S1, were purchased from Thermo Fisher Scientific. Components of the reaction contained 10 μL 2x reaction mix (0.4 mM of each dNTP, 3.2 mM $MgSO_4$), 1.5 μL probe and primer mix (FAM-labelled probe, forward and reverse primers), 0.4 μL SuperScript III RT/Platinum Taq mix, 3.1 μL nuclease free-water, 5 μL RNA template to a final volume of 20 μL. RT-qPCR was performed using a CFXOpus 96 thermal cycler (BioRad, Hercules, CA, USA). The thermal cycling conditions for RT-qPCR assays were as follows: initial incubation at 50 °C for 30 min and initial denaturation at 95 °C for 3 min, followed by 45 cycles of denaturation at 95 °C for 3 s, and the primer annealing and extension reaction at 55 °C for 30 s (acquiring fluorescence in the green filter).

The RT-qPCR assays for SARS-CoV-2 RNA were performed in duplicates and included negative and positive standard controls. To obtain the standard curves, a 10-fold dilution series of standard RNAs was prepared (2019-nCoV_N_Positive Control Cat. PC67102, Norgen).

A Calibration curve for N1 (y = −3.407x + 41.619) and N2 (y = −3.510x + 42.733) showed a linear dynamic, as shown in Fig. 1. The values of efficiency and R^2 were 93.4% and 0.996 for N1 and 85.6% and 0.998 for N2. The limit of detection (LOD) was 10 genome copies for a Ct value of 39.28 ± 0.05 and 39.77 ± 0.58 for N1 and N2, respectively. Table S2 also presents the calibration curves parameters, considering N1 and N2. No amplification inhibition was detected (data not shown). Cycle threshold (Ct) values were used to calculate GC/L in the original sample. Ct values lower than 40 were considered positive for SARS-CoV-2 RNA, as proposed previously [9,10]. Following the protocols described by Rajal et al. [42] and Boxus et al. [43], BRSV RT-qPCR reactions were performed to evaluate the recovery of concentration methods. Recoveries ranging from 20% to 65% were obtained.

2.4. Prevalence estimation

For the data from step 1 (between-day evaluation), prevalence estimates were performed using the SARS-CoV-2 RNA concentration measured in wastewater, considering composite and grab samples, among other parameters, according to the following equations [11,13, 44]:

$$\text{Infected population (N)} = \frac{C_{RNA} \times F}{\alpha \times \beta} \qquad (1)$$

$$\text{Predicted prevalence (\%)} = \frac{N}{\text{Contributing population}} \times 100 \qquad (2)$$

Where;.

C_{RNA} = SARS-CoV-2 RNA concentration measured in composite and grab samples (genome copies.L^{-1}).

F = Wastewater volumetric flow rate (L.d^{-1}).

α = Fecal load (g.$person^{-1}.d^{-1}$).

β = SARS-CoV-2 shedding rate by an infected individual (genome copies.g^{-1}).

The wastewater volumetric flow rate (F) of point 1 was measured *in loco*. In the calculations, the average flow rates of the collection days were considered.

The daily fecal mass (α) produced by humans from low-income countries usually ranges from 75.0 to 520.0 g per person (with an average value of 243.0 ± 130.2 g.$person^{-1}.d^{-1}$), according to Rose et al. [45].

The SARS-CoV-2 shedding rate by an infected individual (β) usually ranges from 6.3×10^5 to 1.3×10^8 genome copies.g^{-1}, according to Kitajima et al. [46] and Gholipour et al. [47].

Thus, the COVID-19 average prevalence was estimated, considering the contributing population of sampling point 1 to be approximately 1,400,000 (Table 1).

2.5. Statistical analysis

In step 1 (between-day evaluation), the one-way analysis of variance (ANOVA) was used to determine whether there were differences among the mean SARS-CoV-2 RNA concentrations of each sampling method, considering a significance level of 0.05 (p-value < 0.05). In addition, descriptive statistics and Pearson's r and Spearman's rho correlation coefficients were used to determine the relationship between the results
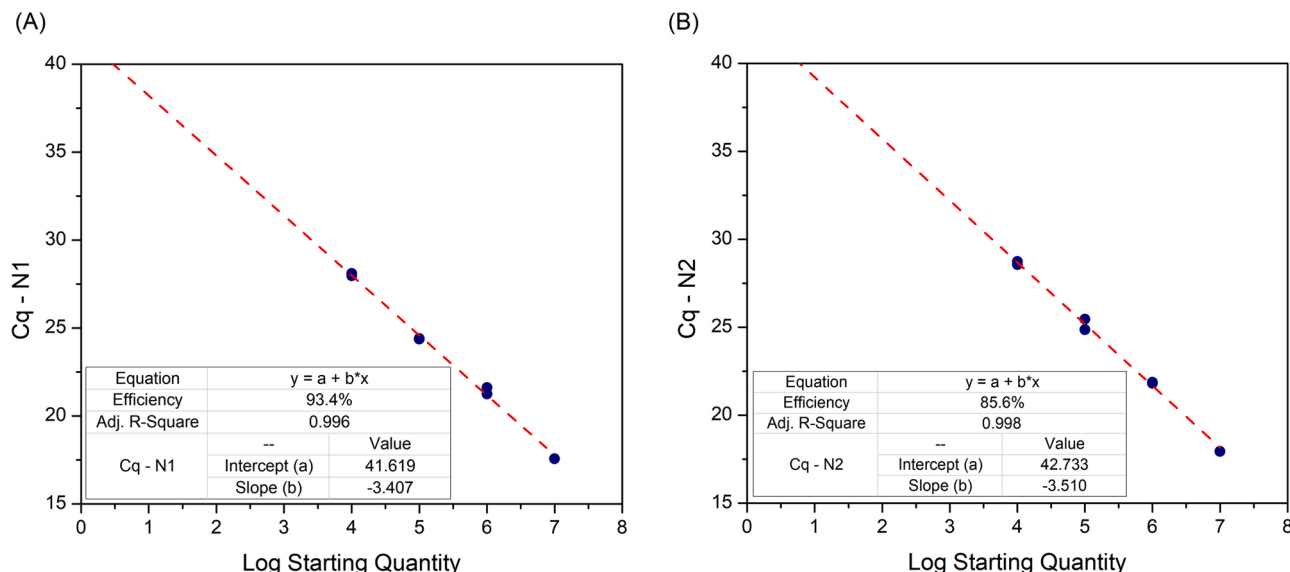
(A)

(B)

Fig. 1. Calibration curves for RT-qPCR N1(A) and N2(B) gene assays.

of composite and grab samplings. As SARS-CoV-2 RNA concentrations in wastewater are log-normally distributed, the statistical analyses were performed with log-transformed data.

The statistical Monte-Carlo approach was incorporated into the calculation of the prevalence estimate (Eq. 1 and Eq. 2) for step 1 data, since parameters such as Fecal load (α) and SARS-CoV-2 shedding rate (β) have large variation, which makes it difficult to interpret the results of the infected population (N) and, consequently, the predicted prevalence. The Monte-Carlo simulation was implemented with 10,000 random samplings of the product of α and β parameters. The α parameter was modeled as a normal distribution with mean $\pm$ standard deviation of 243.0 ± 130.2 g.person^{-1}.d^{-1}, while the β parameter was modeled as a uniform distribution with minimum and maximum values of 6.3×10^5 and 1.3×10^8 genome copies.g^{-1}, respectively [45–47]. Statistical analysis were performed using Origin Pro 8.6, while the Monte-Carlo simulation was implemented in Microsoft Excel.

3. Results and discussion

3.1. Between-day evaluation: long-term monitoring

Composite and grab samples of untreated wastewater from point 1 were analyzed between April 14th, 2021 and August 4th, 2021 (113 days) for SARS-CoV-2 RNA occurrence. Samples with Ct (Cycle threshold) less than 40 were considered positive and had their concentrations determined (genome copies/sample volume) [9].

The RT-qPCR was used to quantify both N1 and N2 gene assays of SARS-CoV-2 RNA in all wastewater samples. The SARS-CoV-2 RNA titers were detected in 88.2% (15/17) and 76.5% (13/17) of composite samples, for N1 and N2 gene assays, respectively, while for the grab samples, the viral genome was detected in 75.0% (12/16) for the two gene assays. In addition to the occurrence percentage, the variability of SARS-CoV-2 RNA concentration for each sampling method was also evaluated (Fig. 2).

For the N1 gene assay, lower variability of SARS-CoV-2 RNA concentration was observed in the grab samples, as evidenced by the smaller height of the box (Fig. 2). For the N2 gene assay, the difference in dataset variability was not clear. Table 2 shows the SARS-CoV-2 RNA concentration descriptive statistic of composite and grab samples by gene assay, complementing the information shown in Fig. 2.

As determined by the one-way analysis of variance (ANOVA), there were no significant differences among the mean SARS-CoV-2 RNA concentrations of each sampling method (p-value > 0.05), considering N1 and N2 gene assays. Therefore, there is good agreement between the SARS-CoV-2 RNA concentration of 24-hour composite samples and grab samples.
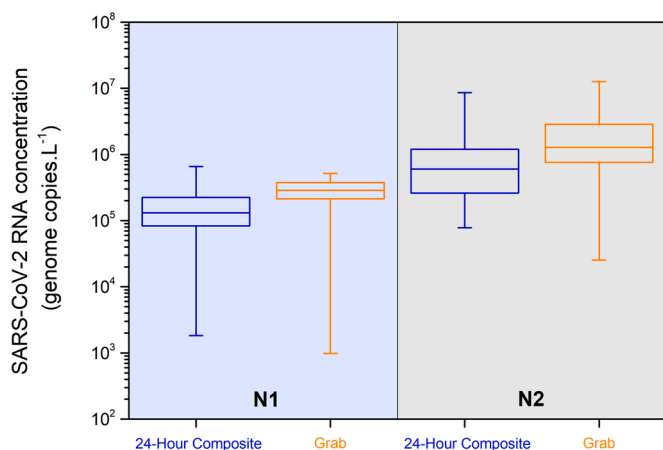

Fig. 2. Box Plot of the SARS-CoV-2 RNA concentration (N1 and N2 assays) per sampling method.

Table 2

Descriptive statistic of SARS-CoV-2 RNA concentration (N1 and N2 assays) per sampling method.

Sampling method	Gene target	SARS-CoV-2 RNA Concentration (log$_{10}$ genome copies.L^{-1})			
		Mean	Median	Minimum	Maximum
Composite	N1	5.3	5.1	3.3	5.8
	N2	6.1	5.8	4.9	6.9
Grab	N1	5.5	5.5	3.0	5.7
	N2	6.4	6.1	4.4	7.1

Differently, Gerrity et al. [40] verified a 10-fold increase in SARS-CoV-2 RNA concentrations in 24-hour composite sampling in comparison to corresponding grab sampling, analyzing primary effluents also from a large WWTP (serving approximately 1 million people). The grab samples of primary effluents corresponded to raw wastewater that arrived at the WWTP between 5 a.m. and 6 a.m., a period of minimum flow rate. However, as attested by Curtis et al. [39], periods of minimum flow rate may have higher concentrations of potentially inhibitory substances for PCR reactions. Therefore, prior assessment of the 24-hour variability of viral load in wastewater is recommended to verify the best moment to perform a grab sampling. In this study, after analyzing the 24-hour variability of SARS-CoV-2 RNA (shown in the next section), we chose to collect grab samples always between 8 a.m. and 10 a.m. For this time interval, there was a lower variability of SARS-CoV-2 RNA titers concentrations and a greater agreement with the average values.

Curtis et al. [39] also observed good agreement between SARS-CoV-2 RNA concentration of 24-hour composite samples and grab samples, with a mean deviation of 1590 copies.L^{-1}. However, unlike the results of this work, the grab sample concentrations were lower than their corresponding composite samples, which can lead to an underestimation of the pandemic severity index. It is worth noting that the study by Curtis et al. [39] was carried out in the early stages of the pandemic, in May 2020, when the accumulated prevalence in the city was approximately 100 cases per 100,000 people. The variation in SARS-CoV-2 RNA is likely to be a function of prevalence. A decrease in variance is expected as the shedding rate increases.

George et al. [48] also found good agreement between SARS-CoV-2 RNA titers concentration of 24-hour composite and grab samples, evaluating a WWTP that serves approximately 48,000 inhabitants (average wastewater flow rate of 150 L.s^{-1}). The grab concentrations ranged from 3.48 ± 0.06–4.47 ± 0.04 log$_{10}$ genome copies.L^{-1}, while the composite concentration was 3.95 ± 0.13 log$_{10}$ genome copies.L^{-1}. Furthermore, there were no negative results (non-detected) in the grab samples dataset. However, the authors observed that as the catchment size decreased, the percentage of negative samples increased, even when the respective composite samples were positive. In addition, differences between grab and composite sample concentrations increased up to two log$_{10}$ units. According to the authors, grab samples can be significantly representative at high-flow sites, such as large WWTPs, but they fail to represent the real conditions at low-flow sites.

Fig. 3 shows viral loads (A) and prevalence estimates (B) for composite and grab samples over 17 weeks of monitoring. The prevalence of SARS-CoV-2 infected individuals (carriers) was estimated using Monte-Carlo simulation with 10,000 random samplings. Hospital admissions and the 14-day moving average of new COVID-19 cases were also plotted. Epidemiological data on COVID-19 in the ABC Region was obtained from the publicly available repository of the São Paulo State Government (available at https://github.com/seade-R/dados-covid-sp).

There is good agreement between the SARS-CoV-2 viral load of 24-hour composite samples and grab samples (Fig. 3(A)). Similarly, the estimates of infected individuals (carriers) and prevalence of the two sampling methods also showed good consensus considering their respective confidence intervals (Fig. 3(B)).
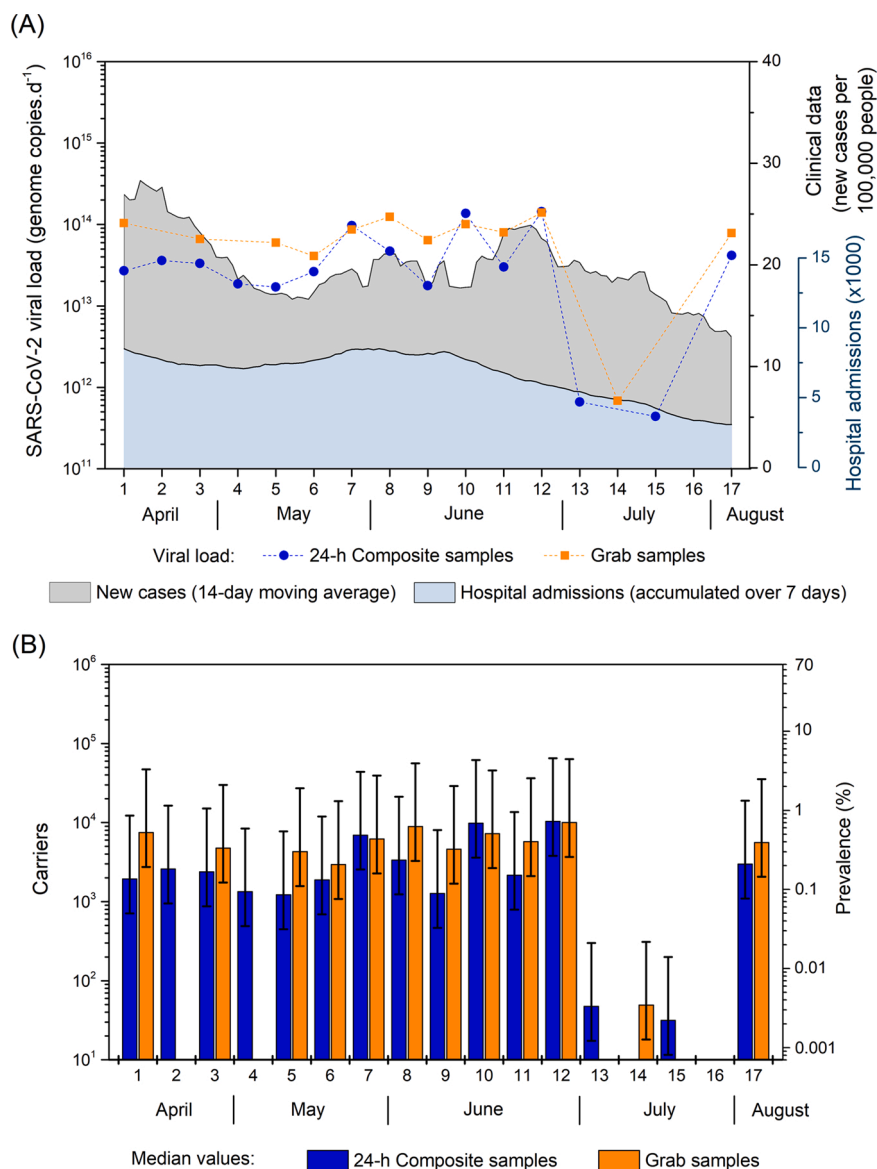
Fig. 3. Viral loads (A) and prevalence estimates (B) for composite and grab samples over 17 weeks of wastewater monitoring. In the lower panel (B), error bars represent the 10th and 90th percentile range in estimates.

However, SARS-CoV-2 RNA titers were not detected in the grab samples of weeks 2, 4, 13, and 15, while the corresponding composite samples indicated the occurrence of the virus. Since grab sampling only represents the exact moment of collection, there is a risk of obtaining false negatives and underestimated viral RNA concentrations [34,49, 50].

On the other hand, at week 14, the presence of the virus was detected only in the grab sample. In certain cases, the grab sampling can be more beneficial, since the bacteriological and pathogenic samples require immediate analysis due to their instability [34]. However, the positive detection for the grab sample and not for the composite sample could also be explained by the effect of the dilution of subsamples collected during periods of SARS-CoV-2 RNA absence.

The viral load of the 24-hour composite samples and grab samples showed no statistically significant correlation with epidemiological data (hospital admissions and 14-day moving average of new COVID-19 cases), as determined by the one-way analysis of variance (ANOVA) considering a significance level of 0.05. The dataset is likely not large enough to allow for trend analysis and comparisons with clinical data. However, this study did not intend to verify and establish these

correlations but aimed to compare and analyze the two most used methods for sanitary sewage sampling in SARS-CoV-2 surveillance studies.

The predicted prevalence, estimated via Monte-Carlo simulation, ranged from 0.001% to 4.7% and from 0.001% to 4.5%, for 24-hour composite and grab samples, respectively, considering a 90% confidence interval. The average values for the two sampling methods were $0.2 \pm 0.2\%$ and $0.4 \pm 0.2\%$, respectively. There was no statistical difference between the means. Therefore, the estimated values of viral load and prevalence from composite and grab samples are statistically equivalent. Differently, Curtis et al. [39] achieved lower viral loads and, consequently, lower prevalence estimates through grab samples. In this case, there is a risk of underestimating the actual SARS-CoV-2 spread, which can lead to neglect of public actions to combat the disease.

The observed COVID-19 prevalence (considering epidemiological/clinical data) in the ABC Region for the same period (April 14th, 2021 – August 4th, 2021) ranged from 0.004% to 0.04%, with an average value of $0.02 \pm 0.01\%$. Regardless of the sampling method, the predicted prevalence values were about ten times higher than the reported prevalence. Previously, long-term monitoring (between June 9th, 2020 and

April 7th, 2021) of this same sampling site resulted in predicted prevalence values equivalent to this study [13]. Wu et al. [10] in Massachusetts, USA, also found prevalence values (0.1 – 5%) higher than those reported from clinical data (about 0.026%). There are many uncertainties in the prevalence estimation model, especially around the SARS-CoV-2 shedding rate which generally ranges from 6.3×10^5 to 1.3×10^8 genome copies.g^{-1}, according to Kitajima et al. [46] and Gholipour et al. [47]. Wölfel et al. [7] indicate that this range may be greater, between $10^{2.67}$ and $10^{7.67}$ genome copies.g^{-1}. Thus, even using Monte-Carlo statistical simulation, the predicted results may differ from the observed data by more than ten times. Both sampling methods can generate representative and useful results for virus tracking, outbreak prediction, and pandemic monitoring. However, grab sampling must be well designed, considering the previously identified peak periods of fecal loading [34]. It is important to emphasize that, regardless of the sampling technique, it is not possible to determine an absolute concentration of the virus in the wastewater [9,13]. The relationship between grab and composite sampling data is shown in Fig. 4. The N1 and N2 data of the positive samples were plotted. Pearson's r and Spearman's rho correlation coefficients were calculated, resulting in 0.83 and 0.78 (p-value < 0.05), respectively.

Pearson's r and Spearman's rho correlation coefficients can range in value from − 1 to + 1. A value of + 1 indicates a perfect positive correlation between two variables (both values rise together) whereas a value of − 1 indicates a perfect negative correlation (while one value increases the other decreases). A value of 0 indicates that there is no relationship between the two variables [51]. The two correlation coefficients were similar and lead to the same conclusion that there is a strong and significant linear relationship between the results from the two sampling methods, considering a significance level of 0.05 [52].

3.2. Within-day evaluation: 24-hour cycles

Fig. 5 shows the daily profile of SARS-CoV-2 RNA concentration wastewater samples from the three sampling points for two different 24-h periods (days 1 and 2). Point 1 represents a large-scale WWTP (Fig. 5 (A)); point 2, a small-scale WWTP (Fig. 5(B)); and point 3, a point in the

Equation	y = a + b*x	
Pearson's r	0.83	
Spearman's rho	0.78	
	-	Value
Parameters	Intercept (a)	1.89
	Slope (b)	0.71

● SARS-CoV-2 RNA concentration
(log$_{10}$ genome copies.L^{-1})

Fig. 4. Correlation between SARS-CoV-2 RNA concentrations obtained from composite and grab sampling.

sewer system (sewer manhole) (Fig. 5(C)). For safety reasons, no samples were collected between 11 p.m. and 5 a.m. at point 3. The figure also shows the results of the coefficient of variation (CV), calculated with log-transformed data.

The wastewater flow rate at point 1 (large-scale WWTP) on day 1 ranged from 1.6 to 3.8 m^3 .s^{-1}, with an average value of 3.2 ± 0.8 m^3 .s^{-1}. On day 2 ranged from 1.6 to 3.6 m^3 .s^{-1}, with an average value of 3.0 ± 0.7 m^3 .s^{-1}. There was no significant difference between the flow rate profile of the two periods analyzed. Similarly, the average SARS-CoV-2 RNA concentrations were not significantly different for the two periods (Fig. 5(A)), although the incidence rate was reduced by half during the second sampling period (day 2).

Still regarding point 1, both 24-hour profiles showed that viral RNA concentrations had less variability and greater agreement with the mean values between 8 a.m. and 10 a.m. For this reason, we chose to perform the grab sampling within this interval in the previous step of the study (between-day evaluation, shown in the previous section). Interestingly, there is a reduction of viral RNA concentrations at the peak flow rate, close to 12 a.m., indicating a possible dilution of the RNA titers shed in the sewer system.

As shown in Fig. 5(B), the wastewater flow rate at the inlet of small-scale WWTP, point 2, is practically constant around 2.0 L.s^{-1}, controlled by a lifting station. Thus, there was low variability in viral load over 24 h, especially for the first period evaluated (day 1), a more severe moment of the pandemic, also evidenced by low CV value. The variation in SARS-CoV-2 RNA is likely to be a function of prevalence. A decrease in variance is expected as the shedding rate increases.

For point 2, the SARS-CoV-2 RNA concentrations in wastewater were significantly lower on day 2. However, both 24-hour profiles showed that viral RNA concentrations had greater consensus with the mean values between 8 a.m. and 10 a.m.

The hourly wastewater flow rates were not measured at point 3 (sewer manhole), due to technical difficulties in accessing the sampling site with measuring instruments. However, the average flow rate was estimated from population data and a per capita wastewater generation of 160 L.person^{-1}.d^{-1}, resulting in 4.5 L.s^{-1}.

As shown in Fig. 5(C), viral load variability was low, especially for the first period evaluated (day 1), resulting in a CV value of 3.0%. In addition, the presence of the fragments of genetic material of SARS-CoV-2 was not detected in only one of the samples (referring to 21:00 on day 2). This result is very interesting since the monitoring points of the sewer system (sewer manhole) require a more practical sampling strategy. As discussed, in these sampling sites it is difficult to install autosamplers, for technical and security reasons. Viral RNA concentrations remained relatively stable and close to the average value between 8 a.m. and 11 a. m., for the two periods analyzed. Thus, grab sampling or even 3-hour semi-composite sampling can yield representative results if they are properly planned.

However, George et al. [48] also evaluating low flow sampling sites (flow rates ranging from 0.8 to 7.0 L/s), found high hourly variability in the SARS-CoV-2 RNA titers concentrations and significant differences between grab samples and their respective composite samples. For the ultra-low-flow sampling site (flow rate of 0.8 L/s), the grab concentrations ranged from 3.44 ± 0.04–7.16 ± 0.02 log$_{10}$ genome copies.L^{-1}, while the composite concentration was 5.81 ± 0.08 log$_{10}$ genome copies.L^{-1}. Unlike our observations, George et al. found [48] that the smaller the catchment area, the greater the hourly variability of concentrations and, therefore, the greater the discrepancies between grab and composite samples.

Fig. 5 also shows the greater variability of data for day 2, a milder moment of the pandemic, especially for sampling points 2 and 3. When there are fewer infected individuals in the contributing area, the viral signal in the wastewater samples may be sporadic throughout the day, being more affected by flow rates variability. Conversely, the viral RNA in wastewater tends to be more stable and less influenced by flow rates variability in areas most affected by the COVID-19 pandemic.
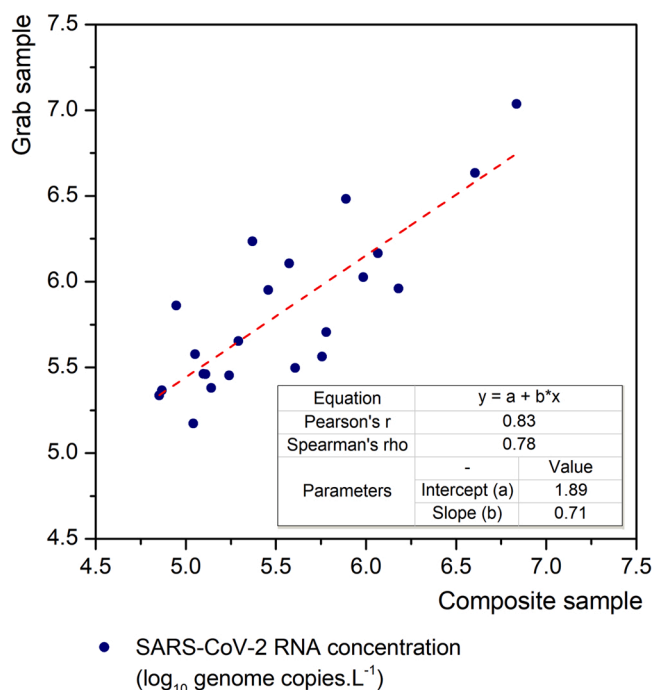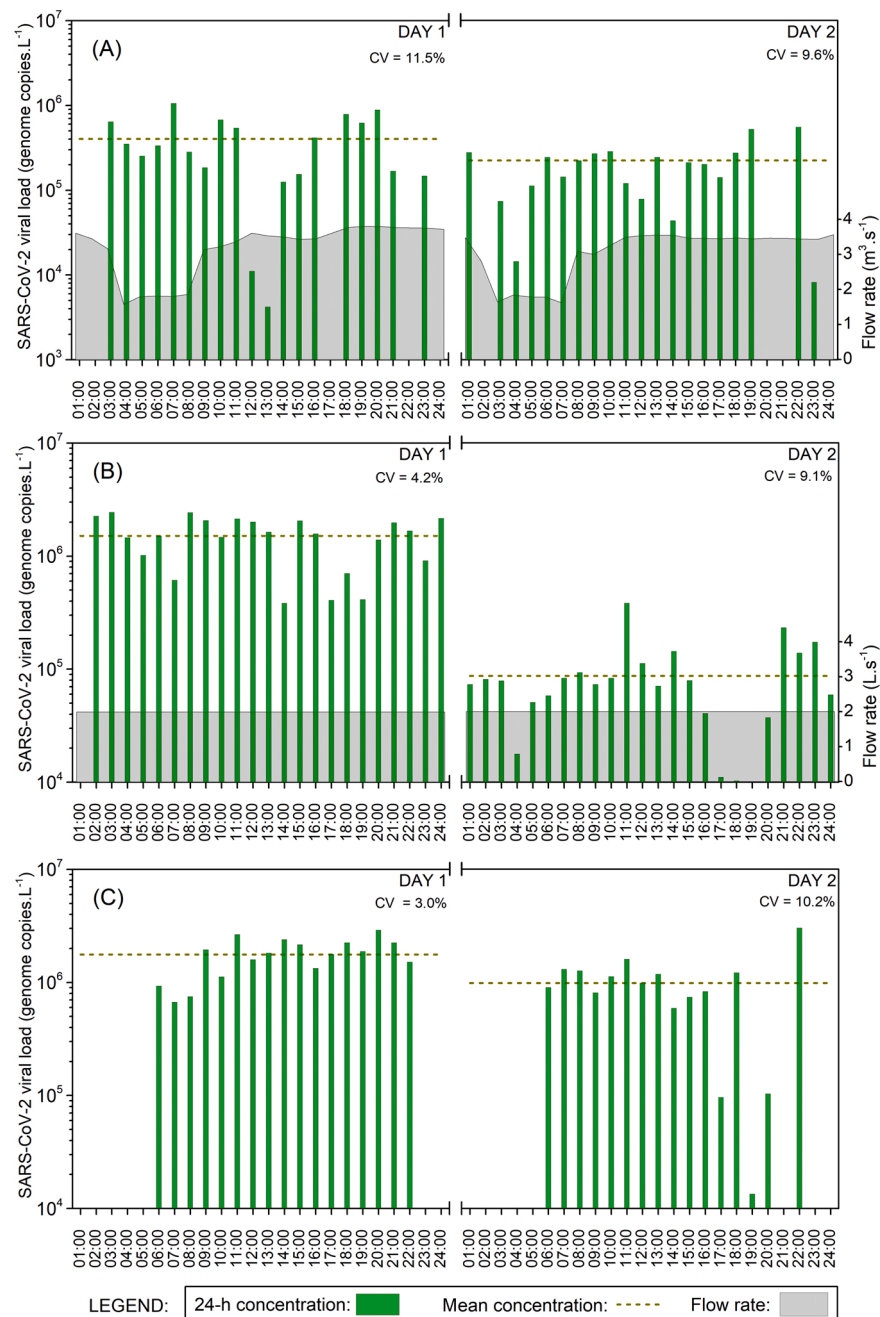
Fig. 5. Daily variation of SARS-CoV-2 RNA concentration in wastewater samples from points 1 (A), 2(B), and 3(C). The first 24-hour cycle (day 1) represents a more severe moment of the pandemic, while the second 24-hour cycle (day 2) represents a milder moment. CV represents the coefficient of variation.

Curtis et al. [39] also evaluated the within-day viral load variability and achieved a CV value of 68.5% (arithmetic data) for grab samples. The SARS-CoV-2 RNA concentrations ranged from 2.5×10^2 to 1.1×10^4 genome copies.L^{-1}. The authors further observed that viral load reductions occurred with a delay of 4–6 h in relation to periods of minimum wastewater flow rate, between 8 a.m. and 11 a.m. However, according to the authors, periods of minimum flow rate should be avoided for sampling, due to the risk of higher concentrations of potentially inhibitory substances for PCR reactions, which can generate false negatives and increase the imprecision of the results.

Bivins et al. [38] recommended carrying out grab sampling during the midday to early evening periods (12 a.m. to 18 p.m.) and avoid early morning periods of minimum flow rates (2–6 a.m.), which are times of low fecal loading. The optimum grab sampling period is the one with the highest fecal loading. The authors suggest monitoring the pepper mild

mottle virus (PMMoV) RNA in the wastewater samples to track the best timing for grab sampling. PMMoV is an elongated rod-shaped virus with a single-stranded genome that occurs in human feces. In addition to using PMMoV as a marker of fecal contribution, other studies have suggested tracking crAssphage (bacteriophage commonly found in human fecal samples), creatinine (breakdown product from muscle and protein metabolism), and total nitrogen. These markers can be used to normalize sewage strength (fecal content per volume of sewage) [53].

Another recent study suggested that performing grab sampling during the peak flow rate period could be an interesting approach to SARS-CoV-2 surveillance in wastewater [54]. However, as attested by Ahmed et al. [35], it is not yet clear if at peak hour of toilet usage we capture the strongest SARS-CoV-2 signal or a more diluted signal. Furthermore, the travel time of sewage from households to the WWTP can range from 2 to 12 h or greater, as in the catchment of our study area. There is also the

contribution of rainwater and stormwater, which can significantly dilute the RNA titers during the heavy rainfalls. In Brazil, domestic sewage and surface run-off (rainwater and stormwater) are collected separately. However, there are many clandestine connections to the sewer network [13].

According to The Water Research Foundation [55], the within-day variation in the concentration of SARS-CoV-2 RNA fragments still remains unknown. In this study, after analyzing the 24-hour variability of SARS-CoV-2 RNA concentrations, we found that the best period to perform grab sampling was between 8 a.m. and 10 a.m. For this time interval, there was less variability of concentrations and greater agreement with the mean values.

Verlicchi et al. [56] evaluated the influence of different sampling strategies in the monitoring of micropollutants in sewage. The sampling methods used were grab sampling, 24-hour time proportional, flow proportional and volume proportional composite sampling. The authors verified that the flow-proportional composite sampling allows more representative measurements, since it showed the smallest ranges of concentrations variability. On the other hand, grab sampling resulted in the greatest range of variability. Evidently, the variability of micropollutants in sewage is quite different from that of microorganisms (viruses, bacteria, and others).

Cornman et al. [57] compared the DNA profiles of different aquatic and semi-aquatic microorganisms in stream water from grab and composite sampling. Although composite sampling resulted in more representative taxonomic profiles, there was no increase in the frequency of detection for specific taxa, whether rare or common overall.

Rodayan et al. [58] evaluated the impact of the sampling strategies used (grab and composite sampling) in monitoring the removal of drugs of abuse during wastewater treatment. The compounds analyzed were consistently detected in the grab and composite samples, that is, those that were not detected in the grab samples were also not detected in the composite samples. Furthermore, the levels of target compounds in grab and composite samples were generally comparable.

Johannessen et al. [59] evaluated the use of grab and composite sampling to monitor phosphorus and suspended solids in the effluent of six small-scale WTTPs. Grab and composite concentrations showed equivalent average values. There was no statistical difference between the results of the two sampling methods, as indicated by the paired two-tailed Student's t-test.

Bertels et al. [53] performed a systematic review of 22 papers on wastewater-based surveillance for SARS-CoV-2 RNA, evaluating different factors, especially associated with the sampling step, which may negatively influence the results. Although the authors recommend flow-proportional composite sampling to track the spread of the virus, they found that 45% of the studies evaluated were performed with grab sampling. The high cost of autosamplers and the high number of monitoring sites usually needed to represent a population/community can make the use of composite sampling unfeasible [59,60]. For this reason, our findings reinforce the relevance of this topic for the scientific community and for the design of future studies and projects on wastewater surveillance. Our results show that both sampling methods can generate representative and useful results for wastewater surveillance. However, grab sampling must be well designed, preferably considering periods of peak fecal load. Evidently, the results shown may be associated with the conditions of the study area, such as pandemic status (infection status), climate, characteristics of the sewage and the sewer system, and others. Thus, it is recommended that comparative studies on sampling strategies for monitoring the RNA of SARS-CoV-2 and other infectious agents be carried out in other regions, with different characteristics, to prove that grab sampling is a viable alternative for virus tracking, outbreak prediction, and pandemic monitoring.

3.3. Towards the implementation of the wastewater surveillance

The ongoing COVID-19 pandemic provides an important opportunity to implement wastewater surveillance, at least at the research level, in different parts of the world. Much progress has been made, but meaningful challenges remain, especially for implementation in emerging countries with limited sources of investment. There are important methodological issues to be elucidated, from advanced methods of virus detection and quantification to the elementary step of wastewater sampling.

This study presents an important discussion on sampling methods, a step that can be one of the bottlenecks for the effective use of wastewater surveillance. Wastewater samples must be representative to enable the correct diagnosis of the monitored population. Thus, sampling strategies must be carefully evaluated, not only regarding the location of monitoring points and the frequency of collection but also regarding the collection method (composite, grab, and passive). Although composite sampling can provide greater representation, its application requires high financial resources, since in most cases automatic samplers are used. There are many difficulties associated with the use of automatic samplers: i) installation and maintenance costs; ii) availability of equipment; iii) access to the sampling site; iv) safety issues (need for permanent care to prevent theft); v) need for a power supply for refrigerated samplers; and vi) excessive depths at sampling sites, often greater than the capacity of the autosampler pump [61].

Wastewater sampling methods must balance the objectives of public health and epidemiological surveillance with available financial and human resources. The objective may simply be to detect the presence of SARS-CoV-2 RNA fragments in a population, which will be especially useful after the pandemic ends for monitoring and preventing new outbreaks (early warning system). However, the objective may also be to quantify the virus in wastewater samples to monitor trends in infection. In this case, the methodological procedures must guarantee greater precision [49].

Regardless, the results of the present study indicated the possibility of using grab sampling, also ensuring the quality of the generated data. The between-day evaluation showed a high correlation between grab and composite samples, with Pearson's r of 0.83 and Spearman's rho of 0.78 (p-value < 0.05). There were no significant differences among the mean SARS-CoV-2 RNA concentrations of each sampling method (p-value > 0.05), considering N1 and N2 gene assays, as determined by the one-way analysis of variance (ANOVA). The average values of predicted prevalence by Monte-Carlo simulation were $0.2 \pm 0.2\%$ and $0.4 \pm 0.2\%$ for composite sampling and grab sampling, respectively.

An absolute comparison between the reported and predicted COVID-19 prevalence is significantly complex [49]. However, as attested by Medema et al. [9], wastewater surveillance can be used to quickly and cost-effectively monitor infection trends in small or large populations. From the detection and quantification of SARS-CoV-2 RNA titers in wastewater samples, it is possible to create an early warning system [62].

4. Conclusions

Wastewater surveillance is a promising and efficient tool with meaningful potential for early warning of outbreaks and infectious disease transmission. By analyzing biomarkers (in this case SARS-CoV-2 RNA) in wastewater sampled at strategic points, disease transmission and spread can be comprehensively monitored in near real-time. This approach is especially useful for emerging countries with limited economical sources and poor epidemiological surveillance systems.

Several studies on the implementation of wastewater surveillance for COVID-19 have been carried out in different locations around the world. Although the potential of the methodology has already been proven, there are many methodological aspects to be elucidated and optimized. It is essential that all methodological steps are standardized, from the definition of sampling strategies to the detection of genetic material. Only in this way we will be able to generate useful results that complement public health and epidemiological surveillance.

In this context, this research is a step towards the improvement of sampling strategies and, consequently, tracking the spread of SARS-CoV-2 RNA from wastewater samples. The results presented showed that it is possible to carry out a representative assessment of a population from grab samples. The within-day evaluation showed that the variability of SARS-CoV-2 RNA over the 24-hour cycles should be considered in defining the best sampling time. In this study, the viral RNA concentrations had greater consensus with the mean values between 8 a.m. and 10 a.m. Therefore, prior evaluation of the 24-hour profile of SARS-CoV-2 RNA in wastewater is recommended for proper implementation of grab sampling, as a viable alternative to composite sampling. The particularities of each region and population must be considered.

CRediT authorship contribution statement

Matheus Ribeiro Augusto: Conceptualization, Data curation, Writing – original draft. **Ieda Carolina Mantovani Claro:** Methodology, Writing – review & editing. **Aline Kaori Siqueira:** Methodology, Writing – review & editing. **Guilherme Santos Sousa:** Methodology, Data curation, Writing – review & editing. **Cláudio Roberto Caldereiro:** Methodology, Writing – review & editing. **Adriana Feliciano Alves Duran:** Methodology, Writing – review & editing. **Taís Browne de Miranda:** Data curation, Writing – review & editing. **Lívia de Moraes Bomediano Camillo:** Data curation, Writing – review & editing. **Aline Diniz Cabral:** Conceptualization, Methodology, Writing – review & editing. **Rodrigo de Freitas Bueno:** Conceptualization, Project administration, Resources, Supervision; Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2022.107478.

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